TITLE OF INVENTION

TRANSFERRIN RECEPTOR GENES OF *MORAXELLA*

FIELD OF INVENTION

The present invention relates to the molecular cloning of genes encoding transferrin receptor (TfR) proteins and, in particular, to the cloning of transferrin receptor genes from *Moraxella (Branhamella) catarrhalis*.

BACKGROUND OF THE INVENTION

Moraxella (Branhamella) catarrhalis bacteria are Gram-negative diplococcal pathogens which are carried asymptomatically in the healthy human respiratory tract. In recent years, *M. catarrhalis* has been recognized as an important causative agent of otitis media. In addition, *M. catarrhalis* has been associated with sinusitis, conjunctivitis, and urogenital infections, as well as with a number of inflammatory diseases of the lower respiratory tract in children and adults, including pneumonia, chronic bronchitis, tracheitis, and emphysema (refs. 1 to 8). (Throughout this application, various references are cited in parentheses to describe more fully the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately preceding the claims. The disclosures of these references are hereby incorporated by reference into the present disclosure). Occasionally, *M. catarrhalis* invades to cause septicaemia, arthritis, endocarditis, and meningitis (refs. 9 to 13).

Otitis media is one of the most common illnesses of early childhood and approximately 80% of all children suffer at least one middle ear infection before the age

of three (ref. 14). Chronic otitis media has been associated with auditory and speech impairment in children, and in some cases, has been associated with learning disabilities. Conventional treatments for otitis media include antibiotic administration and surgical procedures, including tonsillectomies, adenoidectomies, and tympanocentesis. In the United States, treatment costs for otitis media are estimated to be between one and two billion dollars per year.

In otitis media cases, *M. catarrhalis* commonly is co-isolated from middle ear fluid along with *Streptococcus pneumoniae* and non-typable *Haemophilus influenzae*, which are believed to be responsible for 50% and 30% of otitis media infections, respectively. *M. catarrhalis* is believed to be responsible for approximately 20% of otitis media infections (ref. 15). Epidemiological reports indicate that the number of cases of otitis media attributable to *M. catarrhalis* is increasing, along with the number of antibiotic-resistant isolates of *M. catarrhalis*. Thus, prior to 1970, no β-lactamase-producing *M. catarrhalis* isolates had been reported, but since the mid-seventies, an increasing number of β-lactamase-expressing isolates have been detected. Recent surveys suggest that 75% of clinical isolates produce β-lactamase (ref. 16, 26).

Iron is an essential nutrient for the growth of many bacteria. Several bacterial species, including *M. catarrhalis*, obtain iron from the host by using transferrin receptor proteins to capture transferrin. A number of bacteria including *Neisseria meningitidis* (ref. 17), *N. gonorrhoeae* (ref. 18), *Haemophilus influenzae* (ref. 19), as well as *M. catarrhalis* (ref. 20), produce outer membrane proteins which specifically bind human transferrin. The expression of these

proteins is regulated by the amount of iron in the environment.

The two transferrin receptor proteins of *M. catarrhalis*, designated transferrin binding protein 1 (Tbp1) and transferrin binding protein 2 (Tbp2), have molecular weights of 115 kDa (Tbp1) and approximately 80 to 90 kDa (Tbp2). Unlike the transferrin receptor proteins of other bacteria which have an affinity for apotransferrin, the *M. catarrhalis* Tbp2 receptors have a preferred affinity for iron-saturated (i.e., ferri-) transferrin (ref. 21).

M. catarrhalis infection may lead to serious disease. It would be advantageous to provide a recombinant source of transferrin binding proteins as antigens in immunogenic preparations including vaccines, carriers for other antigens and immunogens and the generation of diagnostic reagents. The genes encoding transferrin binding proteins and fragments thereof are particularly desirable and useful in the specific identification and diagnosis of *Moraxella* and for immunization against disease caused by *M. catarrhalis* and for the generation of diagnostic reagents.

There had previously been described in published PCT application WO 97/32380, assigned to Connaught Laboratories Limited, the assignee hereof, the cloning, subcloning and sequencing of nucleic acid molecules encoding transferrin receptor proteins Tbp1 and Tbp2 of certain specific strains of *Moraxella catarrhalis*, namely *M. catarrhlais* strains 4223, Q8 and R1, as well as identifying the deduced amino acid sequences of the encoded Tbp1 and Tbp2 proteins.

WO 97/32380 further describes the construction of expression plasmids for the production of recombinant Tbp1 from *M. catarrhalis* strain 4223 and of recombinant

Tbp2 from *M. catarrhalis* strains 4223 and Q8, the recombinant expression of such proteins in *E. coli*, and the extraction and purification of the expressed Tbp1 and Tbp2 proteins.

SUMMARY OF THE INVENTION

The present invention is directed towards the provision of purified and isolated nucleic acid molecules encoding the transferrin receptor protein Tbp2 of additional strains of *Moraxella catarrhalis*, namely strains M35, 3 and LES1. As in the case of WO 97/32380, the respective genes encoding the Tbp1 and Tbp2 proteins are identified as *tbpA* and *tbpB* genes.

The nucleic acid molecules provided herein are useful for the specific detection of strains of *Moraxella* and for diagnosis of infection by *Moraxella*. The purified and isolated nucleic acid molecules provided herein, such as DNA, are also useful for expressing the *tbp* genes by recombinant DNA means for providing, in an economical manner, purified and isolated transferrin receptor proteins as well as subunits, fragments or analogs thereof.

The transferrin receptor, subunits or fragments thereof or analogs thereof, as well as nucleic acid molecules encoding the same and vectors containing such nucleic acid molecules, are useful in immunogenic compositions for vaccinating against diseases caused by *Moraxella*, the diagnosis of infection by *Moraxella* and as tools for the generation of immunological reagents.

Monoclonal antibodies or mono-specific antisera (antibodies) raised against the transferrin receptor protein, produced in accordance with aspects of the present invention, are useful for the diagnosis of infection by *Moraxella*, the specific detection of

Moraxella (in, for example, *in vitro* and *in vivo* assays) and for the treatment of diseases caused by *Moraxella*.

In accordance with one aspect of the present invention, there is provided a purified and isolated nucleic acid molecule encoding transferrin receptor protein Tbp2 of a strain of *Moraxella*, specifically *M. catarrhalis* strain M35, 3 or LES1.

In one preferred embodiment of the invention, the nucleic acid molecule may encode only the Tbp2 protein of the *Moraxella* strain.

The purified and isolated nucleic acid molecule preferably has a DNA sequence selected from the group consisting of (a) a DNA sequence as set out in Figure 2, 4 or 6 (SEQ ID NOS: 1, 3 or 5) or the complementary DNA sequence thereto; (b) a DNA sequence encoding an amino acid sequence as set out in Figure 2, 4 or 6 (SEQ ID NOS: 2, 4 or 6) or the complementary DNA sequence thereto.

In an additional aspect, the present invention includes a vector adapted for transformation of a host, comprising a nucleic acid molecule as provided herein. Such vector may further comprise expression means operatively coupled to the nucleic acid molecule for expression by the host of the Tbp2 protein of the respective strain of *M. catarrhalis*.

The expression means may include a promoter and a nucleic acid portion encoding a leader sequence for secretion from the host of the transferrin receptor protein. The expression means also may include a nucleic acid portion encoding a lipidation signal for expression from the host of a lipidated form of the transferrin receptor protein or the fragment. The host transformed by the expression vector may be selected from, for example, *Escherichia coli*, *Bordetella*,

Bacillus, *Haemophilus*, *Moraxella*, fungi, yeast or baculovirus and Semliki Forest virus expression systems may be used.

In an additional aspect of the invention, there is provided a transformed host containing an expression vector as provided herein. The invention further includes a recombinant Tbp2 protein of the specific strains of *Moraxella catarrhalis* and producible by the transformed host. Such recombinant Tbp2 proteins have a deduced amino acid sequence selected from the group consisting of those shown in Figure 2, 4 or 6 (SEQ ID NO: 2, 4 or 6).

Such recombinant transferrin receptor protein may be provided in substantially pure form according to a further aspect of the invention, which provides a method of forming a substantially pure recombinant Tbp2 protein of *Moraxella catarrhalis* strain M35, 3 or LES1, which comprises growing the transformed host provided herein to express Tbp2 protein as inclusion bodies, purifying the inclusion bodies free from cellular material and soluble proteins, solubilizing Tbp2 protein from the purified inclusion bodies, and purifying the Tbp2 protein free from other solubilized materials. The substantially pure recombinant transferrin receptor protein is generally at least about 70% pure, preferably at least about 90% pure.

In accordance with another aspect of the invention, an immunogenic composition is provided which comprises at least one active component selected from at least one nucleic acid molecule as provided herein and at least one recombinant protein as provided herein, and a pharmaceutically acceptable carrier therefor or vector therefor. The at least one active component produces an immune response when administered to a host.

The immunogenic compositions provided herein may be formulated as vaccines for *in vivo* administration to a host. For such purpose, the compositions may be formulated as a microparticle, capsule, ISCOM (immunostimulatory complex) or liposome preparation. The immunogenic composition may be provided in combination with a targeting molecule for delivery to specific cells of the immune system or to mucosal surfaces. The immunogenic compositions of the invention (including vaccines) may further comprise at least one other immunogenic or immunostimulating material and the immunostimulating material may be at least one adjuvant or at least one cytokine.

Suitable adjuvants for use in the present invention include (but are not limited to) aluminum phosphate, aluminum hydroxide, QS21, Quil A, derivatives and components thereof, ISCOM matrix, calcium phosphate, calcium hydroxide, zinc hydroxide, a glycolipid analog, an octadecyl ester of an amino acid, a muramyl dipeptide, polyphosphazene, ISCOPREP, DC-chol, DDBA and a lipoprotein.

Advantageous combinations of adjuvants are described in copending United States Patent Applications Nos. 08/261,194 filed June 16, 1994 and 08/483,856, filed June 7, 1995, assigned to the assignee hereof and the disclosures of which are incorporated herein by reference thereto (WO 95/34308).

In accordance with another aspect of the invention, there is provided a method for generating an immune response in a host, comprising the step of administering to a susceptible host, such as a human, an effective amount of the immunogenic composition provided herein. The immune response may be a humoral or a cell-mediated immune response and may provide protection against disease caused by *Moraxella*. Hosts in which protection

against disease may be conferred include primates, including humans.

In a further aspect of the invention, there is provided a live vector for delivery of Tbp2 protein to a host, comprising a vector containing the nucleic acid molecule as described above. The vector may be selected from *Salmonella*, BCG, adenovirus, poxvirus, vaccinia and poliovirus.

The nucleic acid molecules provided herein are useful in diagnostic applications. Accordingly, in a further aspect of the invention, there is provided a method of determining the presence, in a sample, of nucleic acid encoding a transferrin receptor protein of a strain of *Moraxella*, comprising the steps of:

(a) contacting the sample with a nucleic acid molecule as provided herein to produce duplexes comprising the nucleic acid molecule and any nucleic acid molecule encoding the transferrin receptor protein of a strain of *Moraxella* present in the sample and specifically hybridizable therewith; and

(b) determining the production of the duplexes.

In addition, the present invention provides a diagnostic kit for determining the presence, in a sample, of nucleic acid encoding a transferrin receptor protein of a strain of *Moraxella*, comprising:

(a) a nucleic acid molecule as provided herein;

(b) means for contacting the nucleic acid molecule with the sample to produce duplexes comprising the nucleic acid molecule and any such nucleic acid present in the sample and hybridizable with the nucleic acid molecule; and

(c) means for determining production of the duplexes.

The invention further includes the use of the nucleic acid molecules and proteins provided herein as

medicines. The invention additionally includes the use of the nucleic acid molecules and proteins provided herein in the manufacture of medicaments for protection against infection by strains of *Moraxella*.

Advantages of the present invention include:

- an isolated and purified nucleic acid molecule encoding a Tbp2 protein of specific strains of *Moraxella catarrhalis*;
- recombinantly-produced Tbp2 proteins; and
- diagnostic kits and immunological reagents for specific identification of *Moraxella*.

BRIEF DESCRIPTION OF DRAWINGS

The present invention will be further understood from the following description with reference to the drawings, in which:

Figure 1 shows a partial restriction map of the *M. catarrhalis* strain M35 *tbpB* gene;

Figure 2 shows the nucleotide sequence of the *tbpB* gene (SEQ ID NO: 1) and deduced amino acid sequence of the Tbp2 protein of *M. catarrhalis* strain M35 (SEQ ID NO: 2);

Figure 3 shows a partial restriction map of the *tbpB* gene for *M. catarrhalis* strain 3;

Figure 4 shows the nucleotide sequence of *tbpB* gene (SEQ ID NO: 3) and the deduced amino acid sequence of the Tbp2 protein of *M. catarrhalis* strain 3 (SEQ ID NO: 4);

Figure 5 shows a partial restriction map of the *tbpB* genes for *M. catarrhalis* strain LES1;

Figure 6 shows the nucleotide sequence of the *tbpB* gene (SEQ ID NO: 5) and deduced amino acid sequence of the Tbp2 *M. catarrhalis* strain LES1 (SEQ ID NO: 6);

Figure 7 shows an alignment of the Tbp2 proteins from strains 4223 (SEQ ID NO: 7), R1 (SEQ ID NO: 8),

M35 (SEQ ID NO: 2), LES1 (SEQ ID NO: 6), Q8 (SEQ ID NO: 9) and 3 (SEQ ID NO: 4). Dots indicate identical residues and spaces have been introduced to maximize the sequence alignment. Underlining indicates those sequences conserved amongst the *M. catarrhalis* Tbp2 proteins and those from *A. pleuropneumoniae*, *H. influenzae*, *N. gonorrhoeae*, *N. meningitidis* and *P. haemolytica* (SEQ ID NOS: 7, 8 and 9 are disclosed in WO 97/32380);

Figure 8 shows the nucleotide and deduced amino acid sequences of the *M. catarrhalis* strain 4223 *tbpA* - *orf3* - *tbpB* gene locus (SEQ ID NO: 10 - entire gene locus; SEQ ID NO: 11 - *tbpA* coding sequence; SEQ ID NO: 12 - deduced amino acid sequence of TbpA; SEQ ID NO: 13 - *orf3* coding sequence; SEQ ID NO: 14 - deduced amino acid sequence of ORF3; SEQ ID NO: 15 - *tbpB* coding sequence; SEQ ID NO: 7 - deduced amino acid sequence of Tbp2);

Figure 9 shows an alignment of the ORF3 proteins from *M. catarrhalis* strains 4223 (SEQ ID NO: 14) and Q8 (SEQ ID NO: 16). Dots indicate identical residues;

Figure 10 shows a restriction map of clone LEM3-24 the construction of which is described in WO 97/32380 (ATCC deposit No. 97,381 deposited December 4, 1995) showing the location of the *orf3* gene in addition to the *tbpA* and *tbpB* genes of *M. catarrhalis* strain 4223 (cf. Figure 2 of WO 96/32380); and

Figure 11 shows a restriction map of clone SLRD-A the construction of which is described in WO 97/32380 (ATCC deposit No. 97,381 deposited December 4, 1995), showing the locations of the *orf3* gene in addition to the *tbpA* and *tbpB* genes of *M. catarrhalis* strain Q8 (cf. Figure 7 of WO 97/32380).

GENERAL DESCRIPTION OF THE INVENTION

Moraxella catarrhalis strains M35, 3 and LES1 may be conveniently used to provide the purified and isolated nucleic acid, which may be in the form of DNA molecules, comprising at least a portion of the nucleic acid coding for a Tbp2 protein of the strain. Strains 4223, LES1 and M35 are all derived from patients with otitis media while strains 3, R1 and Q8 were from sputum or bronchial secretions.

The *tbpB* genes from *M. catarrhalis* M35, 3 and LES1 were cloned and sequenced herein, following generally the procedures described in WO 97/32380. Strain 3 is a clinical isolate provided by Dr. T. Murphy (State University of New York, Buffalo, New York); strain M35 was obtained from Dr. G.D. Campbell (Louisiana State University, Shreveport, Louisiana) and strain LES1 was obtained from Dr. L. Stanfors (University of Tromso, Finland).

Figures 2, 4 and 6 show the nucleotide sequences of the respective *tbpB* genes (SEQ ID NO: 1, 3 or 5) and deduced amino acid sequence of the Tbp2 protein (SEQ ID NO: 2, 4 or 6) of the *M. catarrhalis* strains M35, 3 and LES1, respectively. Regions of homology are evident between the *M. catarrhalis* Tbp2 amino acid sequences determined herein and those previously determined in WO 97/32380, as shown in the comparative alignment of Figure 7 (SEQ ID NOS: 7, 8, 2, 6, 9 and 4) and between the *M. catarrhalis* Tbp2 amino acid sequences. Underlining in Figure 7 indicates those sequences which are conserved among the *M. catarrhalis* Tbp2 proteins and those of *A. pleuropneumoniae*, *H. influenzae*, *N. gonorrhoeae*, *N. meningitidis* and *P. haemolytica*.

Sequence analysis of the nucleotide acid and amino acid sequences of the Tbp2 proteins described herein

and in WO 97/32380 indicated that at least two families could be identified for *M. catarrhalis tbpB* genes, one comprising strains 4223, R1 and M35 and other comprising strains Q8 and 3, with strain LES1 being equally related to both families. Anti-rTbp2 bactericidal antibody activity (Table 1) correlated with the putative gene families identified by sequencing.

Additional sequence analysis of the entire *M. catarrhalis* strains 4223 and Q8 *tbpA - tbpB* locus gene sequence (Figure 8) identified an intergenic open reading frame termed "*orf3*" (SEQ ID NO: 13, SEQ ID NO: 14, ORF3 amino acid sequence), (see also Figures 10 and 11 for location of orf3). The encoded ORF3 proteins from 4223 and Q8 are 98% identical, as seen from the sequence alignment of Figure 9 (SEQ ID NOS: 14, 16).

Cloned *tbpB* genes may be expressed in *E. coli* to produce recombinant Tbp2 proteins free of other *Moraxella* proteins. These recombinant proteins may be purified and used for immunization.

The Tbp2 proteins provided herein are useful as a diagnostic reagent, as an antigen for the generation of anti-transferrin protein binding antibodies, as an antigen for vaccination against the disease caused by species of *Moraxella* and for detecting infection by *Moraxella* and other such bacteria.

The Tbp2 proteins provided herein may also be used as a carrier protein for haptens, polysaccharides or peptides to make conjugate vaccines against antigenic determinants unrelated to transferrin binding proteins. In additional embodiments of the present invention, therefore, the Tbp2 proteins as provided herein may be used as a carrier molecule to prepare chimeric molecules and conjugate vaccines (including glycoconjugates)

against pathogenic bacteria, including encapsulated bacteria. Thus, for example, glycoconjugates of the present invention may be used to confer protection against disease and infection caused by any bacteria having polysaccharide antigens including lipooligosaccharides (LOS) and PRP. Such bacterial pathogens may include, for example, *Haemophilus influenzae, Streptococcus pneumoniae, Escherichia coli, Neisseria meningitidis, Salmonella typhi, Streptococcus mutans, Cryptococcus neoformans, Klebsiella, Staphylococcus aureus* and *Pseudomonas aeruginosa*. Particular antigens which can be conjugated to Tbp2 proteins and methods to achieve such conjugations are described in U.S. Patent Application No. 08/433,522 filed November 23, 1993 (WO 94/12641), assigned to the assignee hereof and the disclosure of which is hereby incorporated by reference thereto.

In another embodiment, the carrier function of the Tbp2 proteins may be used, for example, to induce an immune response against abnormal polysaccharides of tumour cells, or to produce anti-tumour antibodies that can be conjugated to chemotherapeutic or bioactive agents.

The invention extends to transferrin binding proteins from *Moraxella catarrhalis* for use as an active ingredient in a vaccine against disease caused by infection with *Moraxella*. The invention also extends to a pharmaceutical vaccinal composition containing transferrin binding proteins from *Moraxella catarrhalis* and optionally, a pharmaceutically acceptable carrier and/or diluent.

In a further aspect the invention provides the use of transferrin binding proteins for the preparation of a

pharmaceutical vaccinal composition for immunization against disease caused by infection with *Moraxella*.

It is clearly apparent to one skilled in the art, that the various embodiments of the present invention have many applications in the fields of vaccination, diagnosis, treatment of, for example, *Moraxella* infections and the generation of immunological and other diagnostic reagents. A further non-limiting discussion of such uses is further presented below.

1. Vaccine Preparation and Use

Immunogenic compositions, suitable to be used as vaccines, may be prepared from immunogenic transferrin receptor proteins, analogs and fragments thereof encoded by the nucleic acid molecules as well as the nucleic acid molecules disclosed herein. The vaccine elicits an immune response which produces antibodies, including anti-transferrin receptor antibodies and antibodies that are opsonizing or bactericidal. Should the vaccinated subject be challenged by *Moraxella*, the antibodies bind to the transferrin receptor and thereby prevent access of the bacteria to an iron source which is required for viability. Furthermore, opsonizing or bactericidal anti-transferrin receptor antibodies may also provide protection by alternative mechanisms.

Immunogenic compositions, including vaccines, may be prepared as injectables, as liquid solutions or emulsions. The transferrin receptor proteins, analogs and fragments thereof and encoding nucleic acid molecules may be mixed with pharmaceutically acceptable excipients which are compatible with the transferrin receptor proteins, fragments, analogs or nucleic acid molecules. Such excipients may include water, saline, dextrose, glycerol, ethanol, and combinations thereof. The immunogenic compositions and vaccines may further

contain auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, or adjuvants, to enhance the effectiveness of the vaccines. Immunogenic compositions and vaccines may be administered parenterally, by injection subcutaneously, intradermally or intramuscularly. Alternatively, the immunogenic compositions provided according to the present invention, may be formulated and delivered in a manner to evoke an immune response at mucosal surfaces. Thus, the immunogenic composition may be administered to mucosal surfaces by, for example, the nasal or oral (intragastric) routes. The immunogenic composition may be provided in combination with a targeting molecule for delivery to specific cells of the immune system or to mucosal surfaces. Some such targeting molecules include vitamin B12 and fragments of bacterial toxins, as described in WO 92/17167 (Biotech Australia Pty. Ltd.), and monoclonal antibodies, as described in U.S. Patent No. 5,194,254 (Barber et al). Alternatively, other modes of administration, including suppositories and oral formulations, may be desirable. For suppositories, binders and carriers may include, for example, polyalkalene glycols or triglycerides. Oral formulations may include normally employed incipients such as, for example, pharmaceutical grades of saccharine, cellulose and magnesium carbonate. These compositions may take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain about 1 to 95% of the transferrin receptor proteins, fragments, analogs and/or nucleic acid molecules.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, protective and immunogenic. The quantity to be administered

depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize antibodies, and, if needed, to produce a cell-mediated immune response. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of micrograms of the transferrin receptor proteins, analogs and fragments thereof and/or nucleic acid molecules. Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent administrations. The dosage of the vaccine may also depend on the route of administration and will vary according to the size of the host.

The nucleic acid molecules encoding the transferrin receptor of *Moraxella* may be used directly for immunization by administration of the DNA directly, for example, by injection for genetic immunization or by constructing a live vector, such as *Salmonella*, BCG, adenovirus, poxvirus, vaccinia or poliovirus containing the nucleic acid molecules. A discussion of some live vectors that have been used to carry heterologous antigens to the immune system is contained in, for example, O'Hagan (ref. 22). Processes for the direct injection of DNA into test subjects for genetic immunization are described in, for example, Ulmer et al. (ref. 23).

Immunogenicity can be significantly improved if the antigens are co-administered with adjuvants, commonly used as an 0.05 to 1.0 percent solution in phosphate - buffered saline. Adjuvants enhance the immunogenicity of an antigen but are not necessarily immunogenic themselves. Adjuvants may act by retaining the antigen

locally near the site of administration to produce a depot effect facilitating a slow, sustained release of antigen to cells of the immune system. Adjuvants can also attract cells of the immune system to an antigen depot and stimulate such cells to elicit immune responses.

Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to, for example, vaccines. Intrinsic adjuvants, such as lipopolysaccharides, normally are the components of killed or attenuated bacteria used as vaccines. Extrinsic adjuvants are immunomodulators which are typically non-covalently linked to antigens and are formulated to enhance the host immune responses. Thus, adjuvants have been identified that enhance the immune response to antigens delivered parenterally. Some of these adjuvants are toxic, however, and can cause undesirable side-effects, making them unsuitable for use in humans and many animals. Indeed, only aluminum hydroxide and aluminum phosphate (collectively commonly referred to as alum) are routinely used as adjuvants in human and veterinary vaccines. The efficacy of alum in increasing antibody responses to diphtheria and tetanus toxoids is well established and an HBsAg vaccine has been adjuvanted with alum. While the usefulness of alum is well established for some applications, it has limitations. For example, alum is ineffective for influenza vaccination and inconsistently elicits a cell mediated immune response. The antibodies elicited by alum-adjuvanted antigens are mainly of the IgG1 isotype in the mouse, which may not be optimal for protection by some vaccinal agents.

A wide range of extrinsic adjuvants can provoke potent immune responses to antigens. These include saponins complexed to membrane protein antigens (immune

stimulating complexes), pluronic polymers with mineral oil, killed mycobacteria and mineral oil, Freund's complete adjuvant, bacterial products, such as muramyl dipeptide (MDP) and lipopolysaccharide (LPS), as well as lipid A, and liposomes.

To efficiently induce humoral immune responses (HIR) and cell-mediated immunity (CMI), immunogens are often emulsified in adjuvants. Many adjuvants are toxic, inducing granulomas, acute and chronic inflammations (Freund's complete adjuvant, FCA), cytolysis (saponins and pluronic polymers) and pyrogenicity, arthritis and anterior uveitis (LPS and MDP). Although FCA is an excellent adjuvant and widely used in research, it is not licensed for use in human or veterinary vaccines because of its toxicity.

Desirable characteristics of ideal adjuvants include:

(1) lack of toxicity;

(2) ability to stimulate a long-lasting immune response;

(3) simplicity of manufacture and stability in long-term storage;

(4) ability to elicit both CMI and HIR to antigens administered by various routes, if required;

(5) synergy with other adjuvants;

(6) capability of selectively interacting with populations of antigen presenting cells (APC);

(7) ability to specifically elicit appropriate T_H1 or T_H2 cell-specific immune responses; and

(8) ability to selectively increase appropriate antibody isotype levels (for example, IgA) against antigens.

U.S. Patent No. 4,855,283 granted to Lockhoff et al on August 8, 1989, which is incorporated herein by reference thereto, teaches glycolipid analogues

including N-glycosylamides, N-glycosylureas and N-glycosylcarbamates, each of which is substituted in the sugar residue by an amino acid, as immuno-modulators or adjuvants. Thus, Lockhoff et al. 1991 (ref. 24) reported that N-glycolipid analogs displaying structural similarities to the naturally-occurring glycolipids, such as glycophospholipids and glycoglycerolipids, are capable of eliciting strong immune responses in both herpes simplex virus vaccine and pseudorabies virus vaccine. Some glycolipids have been synthesized from long chain-alkylamines and fatty acids that are linked directly with the sugars through the anomeric carbon atom, to mimic the functions of the naturally occurring lipid residues.

U.S. Patent No. 4,258,029 granted to Moloney, assigned to the assignee hereof and incorporated herein by reference thereto, teaches that octadecyl tyrosine hydrochloride (OTH) functions as an adjuvant when complexed with tetanus toxoid and formalin inactivated type I, II and III poliomyelitis virus vaccine. Also, Nixon-George et al. 1990, (ref. 25) reported that octadecyl esters of aromatic amino acids complexed with a recombinant hepatitis B surface antigen, enhanced the host immune responses against hepatitis B virus.

2. Immunoassays

The transferrin receptor proteins, analogs and/or fragments thereof of the present invention are useful as immunogens, as antigens in immunoassays including enzyme-linked immunosorbent assays (ELISA), RIAs and other non-enzyme linked antibody binding assays or procedures known in the art for the detection of anti-*Moraxella*, transferrin receptor protein antibodies. In ELISA assays, the transferrin receptor protein, analogs and/or fragments corresponding to portions of TfR protein, are immobilized onto a selected surface, for

example, a surface capable of binding proteins or peptides such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed transferrin receptor, analogs and/or fragments, a non-specific protein such as a solution of bovine serum albumin (BSA) or casein that is known to be antigenically neutral with regard to the test sample may be bound to the selected surface. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by non-specific bindings of antisera onto the surface.

The immobilizing surface is then contacted with a sample, such as clinical or biological materials, to be tested in a manner conducive to immune complex (antigen/antibody) formation. This procedure may include diluting the sample with diluents, such as BSA, bovine gamma globulin (BGG) and/or phosphate buffered saline (PBS)/Tween. The sample is then allowed to incubate for from about 2 to 4 hours, at temperatures such as of the order of about 25° to 37°C. Following incubation, the sample-contacted surface is washed to remove non-immunocomplexed material. The washing procedure may include washing with a solution such as PBS/Tween or a borate buffer.

Following formation of specific immunocomplexes between the test sample and the bound transferrin receptor protein, analogs and/or fragments and subsequent washing, the occurrence, and even amount, of immunocomplex formation may be determined by subjecting the immunocomplex to a second antibody having specificity for the first antibody. If the test sample is of human origin, the second antibody is an antibody having specificity for human immunoglobulins and in general IgG. To provide detecting means, the second

antibody may have an associated activity such as an enzymatic activity that will generate, for example, a color development upon incubating with an appropriate chromogenic substrate. Quantification may then achieved by measuring the degree of color generation using, for example, a spectrophotometer.

3. Use of Sequences as Hybridization Probes

The nucleotide sequences of the present invention, comprising the sequence of the transferrin receptor gene, now allow for the identification and cloning of the transferrin receptor genes from any species of *Moraxella*.

The nucleotide sequences comprising the sequence of the transferrin receptor genes of the present invention are useful for their ability to selectively form duplex molecules with complementary stretches of other TfR genes. Depending on the application, a variety of hybridization conditions may be employed to achieve varying degrees of selectivity of the probe toward the other TfR genes. For a high degree of selectivity, relatively stringent conditions are used to form the duplexes, such as low salt and/or high temperature conditions, such as provided by 0.02 M to 0.15 M NaCl at temperatures of between about 50°C to 70°C. For some applications, less stringent hybridization conditions are required such as 0.15 M to 0.9 M salt, at temperatures ranging from between about 20°C to 55°C. Hybridization conditions can also be rendered more stringent by the addition of increasing amounts of formamide, to destabilize the hybrid duplex. Thus, particular hybridization conditions can be readily manipulated, and will generally be a method of choice depending on the desired results. In general, convenient hybridization temperatures in the presence of 50%

formamide are: 42°C for a probe which is 95 to 100% homologous to the target fragment, 37°C for 90 to 95% homology and 32°C for 85 to 90% homology.

In a clinical diagnostic embodiment, the nucleic acid sequences of the TfR genes of the present invention may be used in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including radioactive, enzymatic or other ligands, such as avidin/biotin and digoxigenin-labelling, which are capable of providing a detectable signal. In some diagnostic embodiments, an enzyme tag such as urease, alkaline phosphatase or peroxidase, instead of a radioactive tag may be used. In the case of enzyme tags, colorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with samples containing TfR gene sequences.

The nucleic acid sequences of TfR genes of the present invention are useful as hybridization probes in solution hybridizations and in embodiments employing solid-phase procedures. In embodiments involving solid-phase procedures, the test DNA (or RNA) from samples, such as clinical samples, including exudates, body fluids (e. g., serum, amniotic fluid, middle ear effusion, sputum, bronchoalveolar lavage fluid) or even tissues, is adsorbed or otherwise affixed to a selected matrix or surface. The fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes comprising the nucleic acid sequences of the TfR genes or fragments thereof of the present invention under desired conditions. The selected conditions will depend on the particular circumstances

based on the particular criteria required depending on, for example, the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe etc. Following washing of the hybridization surface so as to remove non-specifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label. It is preferred to select nucleic acid sequence portions which are conserved among species of *Moraxella*. The selected probe may be at least 18bp and may be in the range of about 30 to 90 bp.

4. Expression of the Transferrin Receptor Genes

Plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell may be used for the expression of the transferrin receptor genes in expression systems. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* may be transformed using pBR322 which contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid or phage, must also contain, or be modified to contain, promoters which can be used by the host cell for expression of its own proteins.

In addition, phage vectors containing replicon and control sequences that are compatible with the host can be used as a transforming vector in connection with these hosts. For example, the phage in lambda GEM™-11 may be utilized in making recombinant phage vectors which can be used to transform host cells, such as *E. coli* LE392.

Promoters commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems and other microbial promoters, such as the T7 promoter system as described in U.S. Patent No. 4,952,496. Details concerning the nucleotide sequences of promoters are known, enabling a skilled worker to ligate them functionally with genes. The particular promoter used will generally be a matter of choice depending upon the desired results. Hosts that are appropriate for expression of the transferrin receptor genes, fragments, analogs or variants thereof, may include *E. coli*, *Bacillus* species, *Haemophilus*, fungi, yeast, *Moraxella*, *Bordetella*, or the baculovirus expression system may be used.

In accordance with this invention, it is preferred to make the transferrin receptor protein, fragment or analog thereof, by recombinant methods, particularly since the naturally occurring TfR protein as purified from a culture of a species of *Moraxella* may include trace amounts of toxic materials or other contaminants. This problem can be avoided by using recombinantly produced TfR protein in heterologous systems which can be isolated from the host in a manner to minimize contaminants in the purified material. Particularly desirable hosts for expression in this regard include Gram positive bacteria which do not have LPS and are, therefore, endotoxin free. Such hosts include species of *Bacillus* and may be particularly useful for the production of non-pyrogenic transferrin receptor, fragments or analogs thereof.

EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific

Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Methods of molecular genetics, protein biochemistry and immunology used but not explicitly described in this disclosure and these Examples are amply reported in the scientific literature and are well within the ability of those skilled in the art.

Example 1

This Example illustrates the preparation of chromosomal DNA from *M. catarrhalis* strain M35, following the procedure described in WO 97/32380 for strains 4223 and Q8 (Example 2).

M. catarrhalis isolate M35 was inoculated into 100 ml of BHI broth, and incubated for 18 hr at 37°C with shaking. The cells were harvested by centrifugation at 10,000 x g for 20 min. The pellet was used for extraction of *M. catarrhalis* M35 chromosomal DNA.

The cell pellet was resuspended in 20 ml of 10 mM Tris-HCl (pH 7.5)-1.0 mM EDTA (TE). Pronase and SDS were added to final concentrations of 500 µg/ml and 1.0%, respectively, and the suspension was incubated at 37°C for 2 hr. After several sequential extractions with phenol, phenol:chloroform (1:1), and chloroform:isoamyl alcohol (24:1), the aqueous extract was dialysed, at 4°C, against 1.0 M NaCl for 4 hr, and against TE (pH 7.5) for a further 48 hr with three buffer changes. Two volumes of ethanol were added to the dialysate, and the DNA was spooled onto a glass rod.

The DNA was allowed to air-dry, and was dissolved in 3.0 ml of water. Concentration was estimated, by UV spectrophotometry, to be about 290 µg/ml. This procedure was repeated for the preparation of chromosomal DNA from *M. catarrhalis* strain 3 and LES1.

Example 2

This Example illustrates the construction of a *M. catarrhalis* strain M35 chromosomal library in EMBL3.

A series of *Sau*3A restriction digests of chromosomal DNA from *M. catarrhalis* M35, prepared as described in Example 1, in final volumes of 10 µL each, were carried out in order to optimize the conditions necessary to generate maximal amounts of restriction fragments within a 15 to 23 kb size range. Using the optimized digestion conditions, a large-scale digestion was set up in a 100 µL volume, containing the following: 50 µL of chromosomal DNA (290 µg/ml), 33 µL water, 10 µL 10X *Sau*3A buffer (New England Biolabs), 1.0 µL BSA (10 mg/ml, New England Biolabs), and 6.3 µL *Sau*3A (0.04 U/µL). Following a 15 min. incubation at 37°C, the digestion was terminated by the addition of 10 µL of 100 mM Tris-HCl (pH 8.0)-10 mM EDTA-0.1% bromophenol blue-50% glycerol (loading buffer). Digested DNA was electrophoresed through a 0.5% agarose gel in 40 mM Tris acetate-2 mM $Na_2EDTA.2H_2O$ (pH8.5)(TAE buffer) at 50 V for 6 hr. The region containing restriction fragments within a 15 to 23 kb molecular size range was excised from the gel, and placed into dialysis tubing containing 3.0 ml of TAE buffer. DNA was electroeluted from the gel fragment by applying a field strength of 1.0 V/cm for 18 hr. Electroeluted DNA was extracted once each with phenol and phenol:chloroform (1:1), and

precipitated with ethanol. The dried DNA was dissolved in 5.0 µL water.

Size-fractionated chromosomal DNA was ligated with *Bam*HI-digested EMBL3 arms (Promega), using T4 DNA ligase in a final volume of 9 µL. The entire ligation mixture was packaged into lambda phage using a commercial packaging kit (Amersham), following manufacturer's instructions.

The packaged DNA library was amplified on solid media. 0.1 ml aliquots of *Escherichia coli* strain NM539 in 10 mM $MgSO_4$ (OD_{600} = 0.5) were incubated at 37°C for 15 min. with 15 to 25 µL of the packaged DNA library. Samples were mixed with 3 ml of 0.6% agarose containing 1.0% BBL trypticase peptone-0.5% NaCl (BBL top agarose), and mixtures were plated onto 1.5% agar plates containing 1.0% BBL trypticase peptone-0.5% NaCl, and incubated at 37°C for 18 hr. 3 ml quantities of 50 mM Tris-HCl (pH 7.5)-8 mM magnesium sulfate heptahydrate-100 mM NaCl-0.01% (w/v) gelatin (SM buffer) were added to each plate, and plates were left at 4°C for 7 hr. SM buffer containing phage was collected from the plates, pooled together, and stored in a screwcap tube at 4°C, with chloroform.

Example 3

This Example illustrates screening of the *M. catarrhalis* strain M35 library.

The EMBL3/M35 library, prepared as described in Example 2, was plated onto LE392 cells on YT plates using 0.7% top agar in YT as overlay. Plaques were lifted onto nitrocellulose filters and the filters were probed with oligonucleotide probes labelled with $^{32}P\alpha$-dCTP (Random Primed DNA labeling kit, Boehringer Mannheim). The pre-hybridization was performed in sodium chloride/sodium citrate (SSC) buffer (ref. 27) at

37°C for 1 hour and the hybridization was performed at 42°C overnight. The probes were based upon an internal sequence of 4223 *tbpA*:

I R D L T R Y D P G

(SEQ ID No. 17)

4236-RD 5' ATTCGAGACTTAACACGCTATGACCCTGGC 3'

(Seq ID No 18)

4237-RD 5' ATTCGTGATTTAACTCGCTATGACCCTGGT 3'

(Seq ID No 19).

Putative plaques were re-plated and submitted to second and third rounds of screening using the same procedures.

Phage clone M35-2.3 was found to contain a 13 kb insert of the M35 *tfr* genes. The *tbpB* gene was localized to a 7.5 kb Nhel - Sal I fragment by restriction enzyme and Southern blot analyses and was subcloned into pBR328 for sequence analysis, generating plasmid pLEM40.

A partial restriction map of the M35 *tbpB* gene is shown in Figure 1. The nucleotide and deduced amino acid sequences of the M35 *tbpB* gene are shown in Figure 2. The M35 *tbpB* gene encodes a 706 amino acid protein of molecular weight 76.5 kDa. When the M35 TbpB sequence was aligned with the 4223 TbpB protein (Figure 7), it was found to be 86% identical and 90% similar.

Example 4

This Example illustrates the PCR amplification of the *tbpB* genes from *M. catarrhalis* strains 3 and LES1, following the procedure described in WO 97/32380 for *M. catarrhalis* strain R1.

Oligonucleotide primers were based upon the following sequences, which are found in the intergenic regions surrounding *M. catarrhalis* strain 4223 *tbpB*:

5' GATGGGATAAGCACGCCCTACTT 3' (SEQ ID NO: 20)

sense primer (4940)

5' CCCATCAGCCAAACAAACATTGTGT 3' (SEQ ID NO: 21)
antisense primer (4967)

PCR amplification was performed in buffer containing 100 mM Tris-HCI (pH 8.9), 25 mM KCI, 5 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$. Each 100 µl reaction mixture contained 10 ng of chromosomal DNA from strains 3 and LES1, prepared following the procedure of Example 1, 1 µg each primer, 2.5 U Pwo DNA polymerase (Boehringer Mannheim) and 0.2 mM dNTPs (Perkin Elmer, Foster City, California). The cycling conditions were 25 cycles of 95°C for 30 sec, 45°C for 1.0 min and 72°C for 2.0 min, followed by a 10 min elongation at 72°C. Specific 2.4 kb fragments were amplified and DNA was purified for direct sequencing by agarose gel extraction, using a Geneclean kit (Bio 101 Inc., Vista, California). Plasmid DNA for sequencing was prepared using a Qiagen Plasmid Midi kit (Qiagen, Chatsworth, California). DNA samples were sequenced using an ABI model 373A DNA sequencer using dye terminator chemistry. Oligonucleotide primers of 17 to 25 bases in length were used to sequence both strands of the genes.

Partial restriction maps of the *M. catarrhalis* strains 3 and LES1 *tbpB* genes are shown in Figures 3 and 5 respectively. The nucleotide and deduced amino acid sequences of the strain 3 and LES1 *tbpB* genes are shown in Figures 4 and 6, respectively. The strain 3 *tbpB* gene encodes a 712 amino acid protein of molecular weight 76.9 kDa, which is more closely related to the strain Q8 Tbp2 protein than to the 4223 Tbp2 protein (Figure 7). The Q8 and strain 3 Tbp2 proteins are 71% identical and 79% similar, whereas the 4223 and strain 3 Tbp2 proteins are 51% identical and 64% similar. The strain LES1 *tbpB* gene encodes a 713 amino acid protein

of molecular weight 76.8 kDa which is 63% identical to both the 4223 and Q8 Tbp2 proteins.

From the sequence analysis presented herein and in further consideration of the sequences presented in WO 98/32380, there appear to be at least two gene families which can be identified for *M. catarrhalis tbpB*, one comprising strains 4223, R1 and M35 and the other comprising strains Q8 and 3, with strain LES1 being equally related to both families. This novel finding is similar to that of the *N. meningitidis tbpB* genes which can be divided into two sub-groups (ref. 28). There is limited sequence homology among the amino acid sequences of the *M. catarrhalis* Tbp2 proteins previously identified in WO 98/32380 and in this application and those from other organisms, such as *Actinobacillus pleuropneumoniae*, *H. influenzae*, *N. gonorrhoeae*, *N. meningitidis* and *P. haemolytical* (ref. 29). The homology is scattered in small peptide motifs throughout the sequence and is illustrated by underlining in Figure 7. The conserved LEGGFYG (SEQ ID NO: 22) epitope was present, as found in Tbp2 for other *M. catarrhalis* strains as well as the *H. influenzae* and *N. meningitidis* Tbp2 proteins.

Example 5

This Example illustrates the bactericidal antibody activity of guinea pig anti-4223 rTbp2 and anti-Q8 rTbp2 antibodies, prepared as described in WO 97/32380 (Example 14), and confirmation of the gene families of *tbpB* genes.

The bactericidal antibody assay was performed as described by Yang et al. (ref. 30). Briefly, several *M. catarrhalis* strains were grown to an OD_{578} of 0.5 in BHI medium containing 25 mM EDDA. The bacteria were diluted so that the pre-bleed control plates contained

100 to 300 cfu. Guinea pig anti-rTbp2 antisera and pre-bleed controls, prepared as described in Example 14 of WO 97/32380, were heated to 56°C for 30 min to inactivate endogenous complement and were diluted 1:64 with veronal buffer containing 0.1% BSA (VBS). Guinea pig complement was diluted 1:10 in VBS. Twenty-five µl each of diluted antiserum, bacteria and complement were added to duplicate wells of a 96 well microtiter plate. The plates were incubated at 37°C for 60 min, gently shaking at 70 rpm on a rotary platform. Fifty µl of each reaction mixture were plated onto Mueller Hinton agar plates which were incubated at 37°C for 24 h, then room temperature for 24 h, before the bacteria were counted. Antisera were determined to be bactericidial if $\geq$50% of bacteria were killed compared with negative controls. Each assay was repeated at least twice in duplicate. The assay was performed using both the anti-Tbp2 antisera from both 4223 and Q8 strains against a number of different strains of *Moraxella catarrhalis*. The strains tested are identified and the results obtained are shown in Table 1.

The anti-rTbp2 bactericidal antibody activity shown in Table 1 corelates with the putative gene families identified by sequencing, as described in Example 4. Anti-4223 rTbp2 antibody kills those strains within its own family, i.e. 4223, R1 and M35, while anti-Q8 rTbp2 antibody kills those strains within its family, i.e. Q8, 3 and LES1. The anti-4223 rTbp2 antibody also killed strains VH-9, H-04 and ATCC 25240 indicating that the latter strains may be part of the 4223 family. Strain H-04 was also killed by anti-Q8 rTbp2 antibody.

Example 6

This Example illustrates the sequence analysis of the open reading frame (ORF) within the intergenic region between *M. catarrhalis tbpA* and *tbpB*.

The intergenic region was sequenced for strains 4223 and Q8 and a single open reading frame was identified. This *orf*, identified as *orf3*, was located about 1 kb downstream of *tbpA* and about 273 bp upstream of *tbpB* in each genome (Figure 10 - strain 4223; Figure 11 - strain Q8). The nucleotide and deduced amino acid sequences of the entire 4223 *tbpA - orf3 - tbpB* gene loci are shown in Figure 8. The encoded 4223 and Q8 ORF3 proteins are 98% identical, 512 amino acid proteins, of molecular weight 58.1 kDa and 57.9 kDa, respectively. The alignment of the ORF3 protein sequences is shown in Figure 9.

SUMMARY OF THE DISCLOSURE

In summary of this disclosure, the present invention provides purified and isolated DNA molecules containing transferrin receptor genes of specific strains of *Moraxella catarrhalis*, the sequences of these transferrin receptor genes, and the derived amino acid sequences of the Tbp2 proteins encoded thereby. The genes and DNA sequences are useful for diagnosis, immunization, and the generation of diagnostic and immunological reagents. Immunogenic compositions, including vaccines, based upon expressed recombinant Tbp1 and/or Tbp2, portions thereof, or analogs thereof, can be prepared for prevention of diseases caused by *Moraxella*. Modifications are possible within the scope of this invention.

TABLE I

Bactericidal antibody activity of guinea pig anti-rTbpB antisera

M. catarrhalis strain	Bactericidal Antibody Activity*	
	Anti-4223 rTbp2	Anti-Q8 rTbp2
4223	++	-
M35	++	-
R1	++	-
LES1	-	+
Q8	-	++
3	-	±
VH-9	++	-
H-04	++	++
ATCC 25240	**	-

* killing by antiserum diluted 1:64 compared to negative controls: - indicates 0 to 25% killing; ± indicates 26 to 49%; + indicates 50 to 75%; ++ indicates 76 to 100% killing.

REFERENCES

1. Brorson, J-E., A. Axelsson, and S.E. Holm. 1976. Studies on *Branhamella catarrhalis* (*Neisseria catarrhalis*) with special reference to maxillary sinusitis. Scan. J. Infect. Dis. 8:151-155.

2. Catlin, B.W., 1990. *Branhamella catarrhalis*: an organism gaining respect as a pathogen. Clin. Microbiol. Rev. 3: 293-320.

3. Hager, H., A. Verghese, S. Alvarez, and S.L. Berk. 1987. *Branhamella catarrhalis* respiratory infections. Rev. Infect. Dis. 9:1140-1149.

4. McLeod, D.T., F. Ahmad, M.J. Croughan, and M.A. Calder. 1986. Bronchopulmonary infection due to *M. catarrhalis*. Clinical features and therapeutic response. Drugs 31(Suppl.3):109-112.

5. Nicotra, B., M. Rivera, J.I. Luman, and R.J. Wallace. 1986. *Branhamella catarrhalis* as a lower respiratory tract pathogen in patients with chronic lung disease. Arch.Intern.Med. 146:890-893.

6. Ninane, G., J. Joly, and M. Kraytman. 1978. Bronchopulmonary infection due to *Branhamella catarrhalis* 11 cases assessed by transtracheal puncture. Br.Med.Jr. 1:276-278.

7. Srinivasan, G., M.J. Raff, W.C. Templeton, S.J. Givens, R.C. Graves, and J.C. Mel. 1981. *Branhamella catarrhalis* pneumonia. Report of two cases and review of the literature. Am.Rev. Respir. Dis. 123:553-555.

8. West, M., S.L. Berk, and J.K. Smith. 1982. *Branhamella catarrhalis* pneumonia. South.Med. J. 75:1021-1023.

9. Christensen, J.J., and B. Bruun. 1985. Bacteremia caused by a beta-lactamase producing strain of *Branhamella catarrhalis*. Acta.Pathol. Microbiol. Immunol. Scand. Sect.B 93:273-275.

10. Craig, D.B., and P.A. Wehrle. 1983. *Branhamella catarrhalis* septic arthritis. J. Rheumatol. 10:985-986.

11. Guthrie, R., K. Bakenhaster, R.Nelson, and R. Woskobnick. 1988. *Branhamella catarrhalis* sepsis: a

case report and review of the literature. J.Infect.Dis. 158:907-908.

12. Hiroshi, S., E.J. Anaissie, N.Khardori, and G.P. Bodey. 1988. *Branhamella catarrhalis* septicemia in patients with leukemia. Cancer 61:2315-2317.

13. O'Neill, J.H., and P.W. Mathieson. 1987. Meningitis due to *Branhamella catarrhalis*. Aust. N.Z. J. Med. 17:241-242.

14. Murphy, T.F. 1989. The surface of *Branhamella catarrhalis*: a systematic approach to the surface antigens of an emerging pathogen. Pediatr. Infect. Dis. J. 8:S75-S77.

15. Van Hare, G.F., P.A. Shurin, C.D. Marchant, N.A. Cartelli, C.E.Johnson, D. Fulton, S. Carlin, and C.H. Kim. Acute otitis media caused by *Branhamella catarrhalis*: biology and therapy. Rev. Infect. Dis. 9:16-27.

16. Jorgensen, J.H., Doern, G.V., Maher, L.A., Howell, A.W., and Redding, J.S., 1990 Antimicrobial resistance among respiratory isolates of *Haemophilus influenza, Moraxella catarrhalis,* and *Streptococcus* pneumoniae in the United States. Antibicrob. Agents Chemother. 34: 2075-2080.

17. Schryvers, A.B. and Morris, L.J. 1988 Identification and Characterization of the transferrin receptor from *Neisseria meningitidis*. Mol. Microbiol. 2:281-288.

18. Lee, B.C., Schryvers, A.B. Specificity of the lactoferrin and transferrin receptors in *Neisseria gonorrhoeae*. Mol. Microbiol. 1988; 2-827-9.

19. Schryvers, A.B. Characterization of the human transferrin and lactoferrin receptors in *Haemophilus influenzae*. Mol. Microbiol. 1988; 2: 467-72.

20. Schryvers, A.B. and Lee, B.C. (1988) Comparative analysis of the transferrin and lactoferrin binding proteins in the family *Neisseriaceae*. Can. J. Microbiol. 35, 409-415.

21. Yu, R. and Schryvers, A.B., 1993. The interaction between human transferrin and transferrin binding protein 2 from *Moraxella (Branhamella) catarrhalis*

differs from that of other human pathogens. Microbiol. Pathogenesis, 15:433-445.

22. O'Hagan, 1992. Clin. Pharmokinet. 22:1.

23. Ulmer et al., 1993. Curr. Opinion Invest. Drugs 2: 983-989.

24. Lockhoff, O., 1991. glycolipds as immunomoclutators: Synthesis and properits. Chem. Int. Ed. Engl. 30: 1611-1620.

25. Nixon-George, 1990. J. Immunol. 14: 4798-4802.

26. Wallace, R.J. Jr., Nash, D.R., and Steingrube, V.A. 1990. Antibiotic susceptibilites and drug resistance in *Moraxella (Branhaemella) catarrhalis*. Am. J. Med. 88 (5A): 465-50S.

27. F.M. Ausubel et al., Short protocols in Molecular Biology, Greene Publishing Associates and John Wiley and Sons.

28. Legrain, M., V. Mazarin, S.W. Irwin, B. Bouchon, M-J. Quentin-Millet, E. Jacobs, and A.B. Schryvers. 1993, Cloning and characterization of Neisseria meningitidis genes encoding the transferrin-binding proteins Tbp1 and Tbp2. Gene 130: 73-80.

29. Ogunnariwo, J.W., Woo, T.K.W., Lo, R.Y.C., Gonzalez, G.C., and Schryvers, A.B. Characterization of the Pasteurella haemolytica transferrin receptor genes and the recombinant receptor proteins. Microb. Pathog. 23:273-284 (1997).

30. Yang, Y.P., Myers, L.E., McGuinness, U., Chong, P., Kwok, Y., Klein, M.H. and Harkness R.E. The major outer membrane protein, C.D, extracted from Moraxella (Branhamella) catarrhalis is a potential vaccine antigen that induces bactericidal antibodies. FEMS Immun. Med. Microbiol. 17:187-199 (1997).